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IRX1 ameliorates sepsis-induced acute kidney injury in mice by promoting CXCL14

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Abstract
Background: Sepsis-induced acute kidney injury is a general critical complication having high relevance to kidney inflammation. In spite of advances in clinical and critical care, the specific and effective therapies for acute kidney injury are still insufficient. The present study aimed to investigate the protective effect of Iroquois homeobox genes (*IRX*) on sepsis-induced kidney dysfunction in mice.
Methods: In order to gain insight into sepsis-related actions in acute kidney injury, the cecal puncture-induced kidney injury animal model was established. The hematoxylin and eosin staining was used to measure the pathology of kidney tissues. The kidney function-related biomarkers, including neutrophil gelatinase-associated lipocalin, creatinine, kidney injury molecule-1, blood urea nitrogen, and inflammatory cytokines, which included tumor necrosis factor α, interleukin 1β (IL-1β), IL-6, and monocyte chemotactic protein 1, were detected by automated biochemical analyzer or their corresponding test kits. The protein expression was measured using Western blot analysis, and the apoptotic rate of kidney tissue was measured by terminal deoxynucleotidyl transferase dUTP nick end labeling assay.
Results: The present study revealed the protective ability of *IRX1* in sepsis-induced acute kidney injury. This study also determined the potential mechanism of *IRX1* on sepsis-induced inflammatory response and cell apoptosis. Finally, it highlighted that *IRX1* exerted a protective influence on CLP-induced acute kidney injury by suppressing the activation of chemokine (C-X-C motif) ligand 14 (CXCL14).
Conclusion: To conclude, the results suggest that overexpression of *IRX1* could promote survival rate and suppress the CLP-induced apoptosis, inflammatory response, and kidney dysfunction through the activation of CXCL14. *IRX1* and CXCL14 are essential to elucidate the mechanism of acute kidney injury. These findings may help to identify the promising targets for clinical sepsis therapy.

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Introduction

Sepsis is caused by a dysregulated host response to infection. It is thought to be a systemic inflammatory response that leads to extensive tissue damage and multi-organ failure.[1] It has been the main cause of deaths in intensive care units (ICUs). Acute kidney injury is one of the most serious and common complications in the progression of sepsis.[2,3] In spite of advances in clinical and intensive care, specific and effective therapies for acute kidney injury are still insufficient. According to recent statistics, the incidence of acute kidney injury is as high as 70%, and acute renal failure occurs in approximately 5% of patients.[4] Therefore, development of new and effective strategies for sepsis-induced acute kidney injury is vital and required urgently.

Chemokine (C-X-C motif) ligand 14 (CXCL14) is a relatively new CXC chemokine that is frequently expressed in breast, kidney, and other epithelial tissues.[5,6] A previous study revealed that CXCL14 expression in the kidney was significantly reduced after 12 h of cecal puncture (CLP).[7] An investigation showed an inverse correlation between renal CXCL14 expression and markers of acute kidney injury, including serum creatinine (Scr) and renal neutrophil gelatinase-associated lipocalin (NGAL).[7] In addition, the overexpression of CXCL14 could inhibit the production of inflammation-related cytokines (tumor necrosis factor α [TNF-α], interleukin 6 [IL-6], and IL-1β) and renal neutrophil gelatinase-associated lipocalin expression in the kidney as well as serum creatinine levels.[7,8] In *in vivo* and *in vitro* studies, CXCL14 overexpression could reduce macrophage M1 polarization but increase macrophage M2 polarization.[7,9,10] Previous studies have suggested that CXCL14 overexpression may attenuate sepsis-induced kidney injury by down-regulating macrophage-derived cytokine production.[7] Iroquois homeobox genes (*IRX*) are members of the homeobox-containing transcription factors that play a major role in embryonic development in both vertebrates and invertebrates.[11,12] The *IRX* gene was first discovered in a mutagenesis screening in Drosophila and later in other animals.[13] Numerous studies have indicated that *IRX1* is implicated in lung, brain, kidney, and skeletal joints development; however. there has been limited focus on the relevance of its functioning and cellular mechanisms to development.[14,15] Studies have also shown that *IRX1* hypermethylation inhibits CXCL14 expression and promotes heart failure.[16,17] However, the effect of *IRX1* on the sepsis-induced acute kidney injury remains elusive.

Therefore, in this study, an animal model of acute kidney injury was established to investigate whether *IRX1* could exert its protective effects by activating CXCL14 expression. The novelty of this study is high because it is the first evidence indicating that *IRX1* and CXCL14 are essential for elucidating the mechanisms of acute kidney injury. These findings may help to identify the promising targets for clinical sepsis therapy.

Methods

Animal model

Adult BALB/c mice weighing 20–25 g (aged 8 weeks) were housed under 12 h light and 12 h dark cycle and had free access to diet and tap water. All experimental procedures involving animals were approved by the Ethics Committee of the Affiliated Hospital of North Sichuan Medical College, and were conducted in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. The cecal ligation and puncture (CLP) surgery was performed to trigger sepsis-induced kidney injury as described previously.[18] Mice were randomly divided into the following seven groups: sham-operated group; CLP-operated group; adeno-associated virus (AAV)-IRX1 injection with CLP (CLP + AAV-IRX1) group; AAV-empty injection with CLP (CLP + AAV-empty) group; short hairpin negative control lentiviral vector (sh-NC) injection with CLP + AAV-IRX1 (CLP + AAV-IRX1 + sh-NC) group; sh-CXCL14 injection with CLP + AAV-IRX1 (CLP + AAV-IRX1 + sh-CXCL14) group, and sh-NC injection with CLP + AAV-empty (CLP + AAV-empty + sh-NC) group. CLP surgery was performed after 2 weeks of sh-CXCL14 or sh-NC injection.

Western blot analysis

Kidney tissues were collected and homogenized, then using radioimmunoprecipitation assay (RIPA) buffer these were centrifuged (13,000 g, 4°C, 15 min). Protein samples, 20 μg, were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The primary antibodies against *IRX1* (1:2000; Thermo Fisher Scientific, San Jose, CA, USA), Bcl-2-associated X (Bax, 1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), B-cell lymphoma-2 (Bcl-2, 1:5000; Abcam, Cambridge, USA), CXCL14 (1:2000; Thermo Fisher Scientific), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:10,000; Abcam) overnight at 4°C. Horseradish peroxidase-labeled secondary antibody was used to incubate the membrane at 37°C for 1 h. Proteins on the membrane were visualized by enhanced chemiluminescence (Sigma-Aldrich, St. Louis, MO, USA). GAPDH was used as an internal control.

Enzyme-linked-immunosorbent serologic assay (ELISA)

The levels of inflammatory cytokines, including TNF-α, IL-1β, IL-6, monocyte chemotactic protein 1 (MCP-1), in blood were measured using ELISA according to the manufacturer's protocol (DY410-05, MLB00C, DY479-05; R&D Systems, Minneapolis, MN, USA) at room temperature.

Hematoxylin and eosin (H&E) staining

Mice were sacrificed and kidney tissues were perfused with 10% paraformaldehyde. The kidney tissues were embedded in paraffin, and cut into 5-μm-thick sections. The slices were stained with H&E (Abcam).[19] The histological changes of the kidney were examined under a light microscope (Olympus Tokyo, Japan), and images were taken with a microscope-attached camera.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The cell apoptotic ratio in mice kidney tissue (3 μm) was measured by TUNEL staining kit (Sigma-Aldrich) according to the manufacturer's protocol. The slices were treated with a TUNEL reaction mixture for 1 h at 37°C and then added in converter-peroxidase for 30 min. After air-drying, images were captured for the calculation of TUNEL-positive cells.[20]

Determination of kidney function

Blood samples were collected with centrifugation at 5000 rpm for 8 min. The levels of serum cystatin C (ScysC), blood urea nitrogen (BUN), and Scr in serum were measured by Olympus AU400 automated biochemical analyzer (Olympus). The urine levels of kidney injury molecule-1 (KIM-1) and NGAL were determined using commercially available ELISA kits (DY1817, MLCN20; R&D System) in accordance with the manufacturer's protocol.

Statistics

All experiments were conducted in triplicate and analyzed using GraphPad Prism Software 7 (La Jolla, CA, USA). Quantitative data were presented as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) and *t*-test were used for data comparison. A statistically significant difference was defined as $P < 0.05$.

Results

IRX1 ameliorates CLP-induced kidney dysfunction in mice

The CLP surgery-caused kidney injury is a widely used animal model for studying acute kidney injury. In order to study the actions of *IRX1* in acute kidney injury, an animal model was established by sepsis in CLP-operated mice. Before CLP procedure, the mice were transfected with two different AAVs: AAV-empty and AAV-IRX1. The IRX1 protein expression was determined by Western blotting analysis (Figure 1A). Densitometric analysis of the bands of Western blots showed an obviously decreased expression of *IRX1* after CLP procedure and increased expression of *IRX1* in the CLP + AAV-IRX1 group as compared to the sham group and the CLP + AAV-IRX1 group, respectively (Figure 1A). In order to investigate the protective effects of *IRX1* on CLP-induced acute kidney injury, the related biomarkers were discovered. As shown in Figure 1B, the expressions of BUN, Scr, ScysC, KIM-1, and NGAL had significantly gone up after CLP procedure in the mice model of acute kidney injury, and the CLP + AAV-IRX1 group had obviously reversed the CLP-induced effects. Together, these results demonstrated that *IRX1* overexpression ameliorated kidney function in the mice model of acute kidney injury.

IRX1 has a protective role against CLP-induced kidney injury in mice

In order to evaluate the protective role of *IRX1* in CLP-induced kidney injury, H&E staining of the kidney

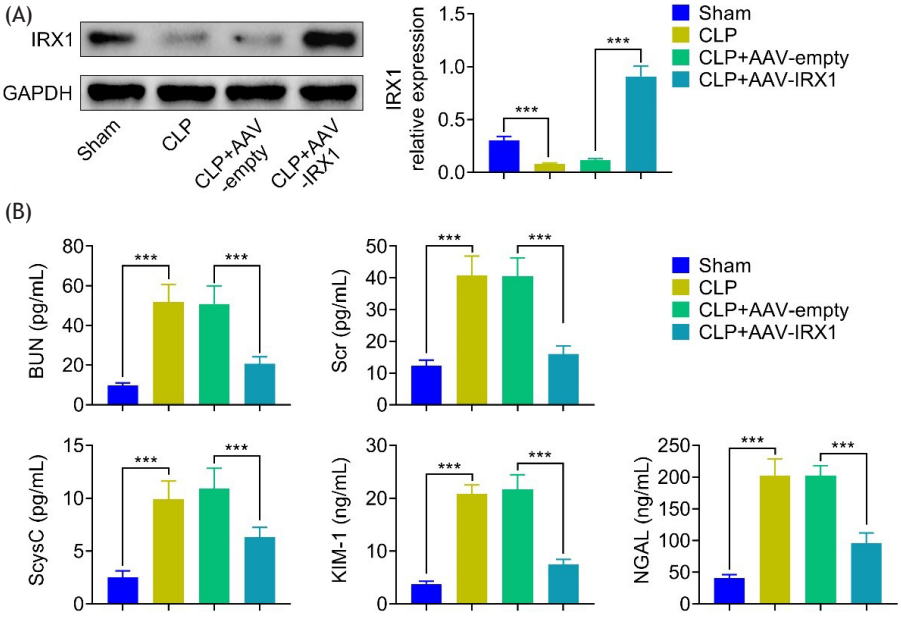

Figure 1 Effects of *IRX1* on the CLP-induced kidney dysfunction in mice. (A) Mice were injected with AAV-empty or AAV-IRX1 before CLP procedure. The IRX1 protein expression was determined by Western blot analysis. (B) The levels of Scr, ScysC, and BUN were measured by an automatic biochemical analyzer. The levels of NGAL and KIM-1 were measured by commercial kits. ***$P < 0.005$ vs. the sham or IRX1 + AAV-empty group. Data were expressed as mean ± SEM.

tissue samples was performed. There were no significantly abnormal pathological changes in the kidney tissue of the sham group. After CLP surgery, the structure of the glomerular was vague, the renal tissue was obviously edematous, narrowing of the renal cystic lumen could be observed, and a numerous number of inflammatory cell infiltrations were determined in mice. In addition, the renal tubules were dilated and deformed, the tubule epithelium was shed, and the brush border had disappeared in the CLP group. Importantly, treatment of AAV-IRX1 attenuated abnormal histological phenomena in the CLP + AAV-empty group (Figures 2A and B). As shown in Figure 2C, the survival rate was evidently raised in the CLP group compared to the sham group. Meanwhile, the survival rate of CLP-operated mice was remarkably suppressed after treatment with AAV-IRX1. These results indicated that *IRX1* might relieve kidney injury caused by sepsis and improve the survival rate the in mice model of acute kidney injury.

IRX1 reduces CLP-induced inflammatory responses in mice kidney

In order to determine the conservatory effect of *IRX1* on CLP-induced inflammatory response in the kidney, the mice were transfected with AAV-empty or AAV-IRX1. Expressions of pro-inflammatory cytokines, including IL-6, IL-1β, (MCP-1, and TNF-α, in CLP-operated mice were also significantly higher than that in the sham group (Figure 3). Compared with the CLP + AAV-empty group, treatment of AAV-IRX1 attenuated the expressions of inflammatory cytokines (Figure 3). These results implied that *IRX1* could be involved in the regulatory processes of CLP-induced inflammatory response in mice.

IRX1 attenuates CLP-induced cell apoptosis in mice kidney

After CLP procedure, kidney injury-induced DNA fragmentation increased the number of TUNEL-positive cells in the

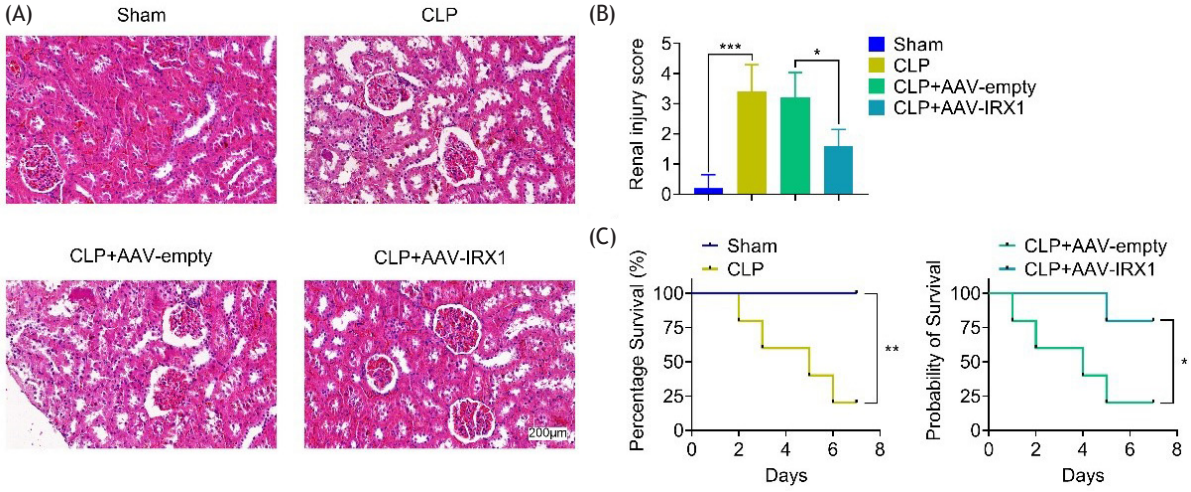

Figure 2 Effects of *IRX1* on the CLP-induced kidney injury in mice. (A) H&E staining was performed to measure the histopathological changes. (B) Quantitative result of histological injury on (A). (C) Kaplan-Meier survival curves showed the survival rate of sepsis mice model in different treatment groups. *P < 0.05, **P < 0.01, and ***P < 0.005 vs. the sham or IRX1 + AAV-empty group. Data were expressed as mean ± SEM.

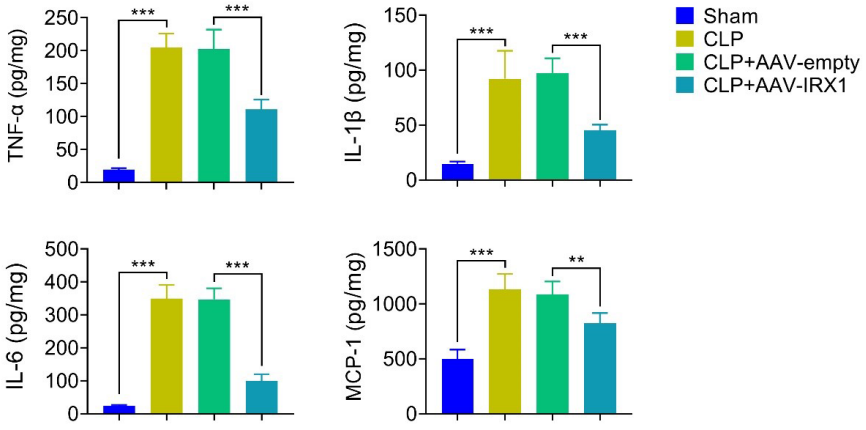

Figure 3 Effects of *IRX1* on CLP-induced inflammatory response in mice. The expression levels of inflammatory cytokines in blood, including TNF-α, IL-6, MCP-1, and IL-1β were measured by commercial ELISA kits. **P < 0.01 and ***P < 0.005 vs. the sham or IRX1 + AAV-empty group. Data were expressed as mean ± SEM.

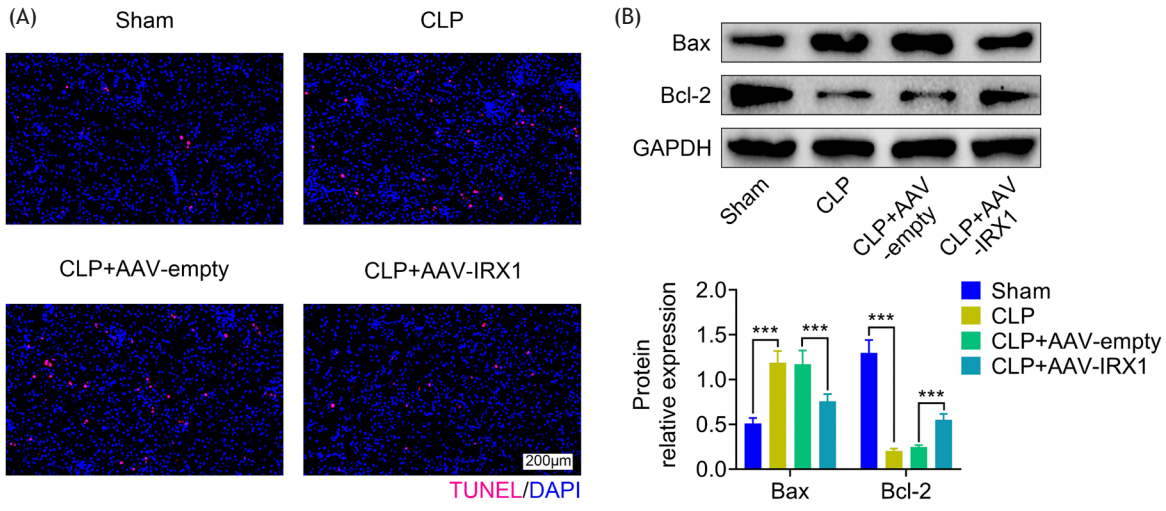

Figure 4 Effects of *IRX1* on CLP-induced cell apoptosis in mice. (A) Cell apoptosis in kidney tissue was evaluated by the TUNEL assay. (B) The protein expressions of Bax and Bcl-2 were examined by Western blot analysis. ***P < 0.005 vs. the sham or IRX1 + AAV-empty group. Data were expressed as mean ± SEM.

CLP group compared to that in the sham group. Conversely, the number of TUNEL-positive cells was obviously suppressed in the CLP + AAV-IRX1 group as compared to that in the CLP + AAV-empty group (Figure 4A). Western blot analysis was performed to measure the expressions of apoptosis-related proteins, Bax and Bcl-2. As presented in Figure 4B, the protein expression level of Bax in the CLP group was markedly increased compared to that in the sham group. However, the protein expression level of Bax was obviously reduced in the CLP + AAV-IRX1 group compared to that in the CLP + AAV-empty group. Moreover, the protein expression level of Bcl-2 was significantly decreased compared to that in the sham group. However, the protein expression of Bcl-2 was obviously increased in the CLP + AAV-IRX1 group compared to that in the CLP + AAV-empty group (Figure 4B). Together, these data support a protective role of *IRX1* in mediating CLP-induced cellular apoptosis.

IRX1 exerts a protective effect against CLP-induced kidney injury in mice via CXCL14 regulation

In order to investigate further the mechanism of *IRX1* underlying CLP-induced acute kidney injury, the present study explored the regulatory role of *IRX1* in the activation of CXCL14 in CLP-induced kidney injury in mice. Compared to the sham group, the CXCL14 expression was remarkably suppressed in mice upon CLP procedure, but treatment with AAV-IRX1 reversed this effect (Figure 5A). After CLP procedure, mice were co-transfected with AAV-IRX1 lentivirus and sh-NC or sh-CXCL14. As shown in Figure 5B, expressions of BUN, Scr, ScysC, KIM-1, and NGAL were significantly decreased after CLP + AAV-IRX1 + sh-NC treatment compared to that in the CLP + AAV-empty + sh-NC group. Conversely, sh-CXCL14 treatment obviously reversed the protective effects of AAV-IRX1. Moreover, CLP + AAV-IRX1 treatment decreased the enhancement inflammatory cytokines (TNF-α, IL-6, IL-1β, and MCP-1) caused by CLP, but were reversed in the CLP + AAV-IRX1 + sh-CXCL14 group. Similarly, CLP + AAV-IRX1 + sh-CXCL14 treatment markedly enhanced Bax expression but suppressed Bcl-2 expression, compared to that in the CLP + AAV-IRX1 + sh-NC group. These results highlighted that *IRX1* exerted a protective influence on CLP-induced acute kidney injury by suppressing CXCL14 activation.

Discussion

Sepsis is a serious disease caused by bacterial infection.[1] The abnormal inflammatory response of organism exacerbates infectious condition and leads to multiple organ failure and organ fibrosis. In sepsis, acute kidney injury is a common critical complication, and is associated with kidney inflammation.[21] It is mainly characterized by elevated serum urea, secondary to the induction of uremic symptoms, and causes acute renal dysfunction.[22-24] Numerous studies have indicated that acute kidney injury has complex mechanism, including abnormal biomarkers of kidney injury, such as insulin-like growth factor-binding protein 7 (IGFBP7), NGAL, and Netrin-1 protein.[25,26] Even though these biomarkers seem to be more beneficial for early discovery of acute kidney injury, more studies are required to confirm their relevance in clinical diagnosis. Limited data are available regarding functional association between the *IRX1* gene and acute kidney injury. Understanding the functioning of genes in kidney cells is an important approach to study renal dysfunction caused by sepsis. The present study is intended to reveal the protective ability of *IRX1* in sepsis-induced acute kidney injury using a CLP-operated animal model of sepsis. This study was first to investigate the impact of *IRX1* on histopathological injury and kidney dysfunction in sepsis-induced acute kidney injury in mice. This study also identified the potential mechanisms of *IRX1* on sepsis-induced inflammatory response and cell apoptosis. Although inflammation is a critical defense mechanism

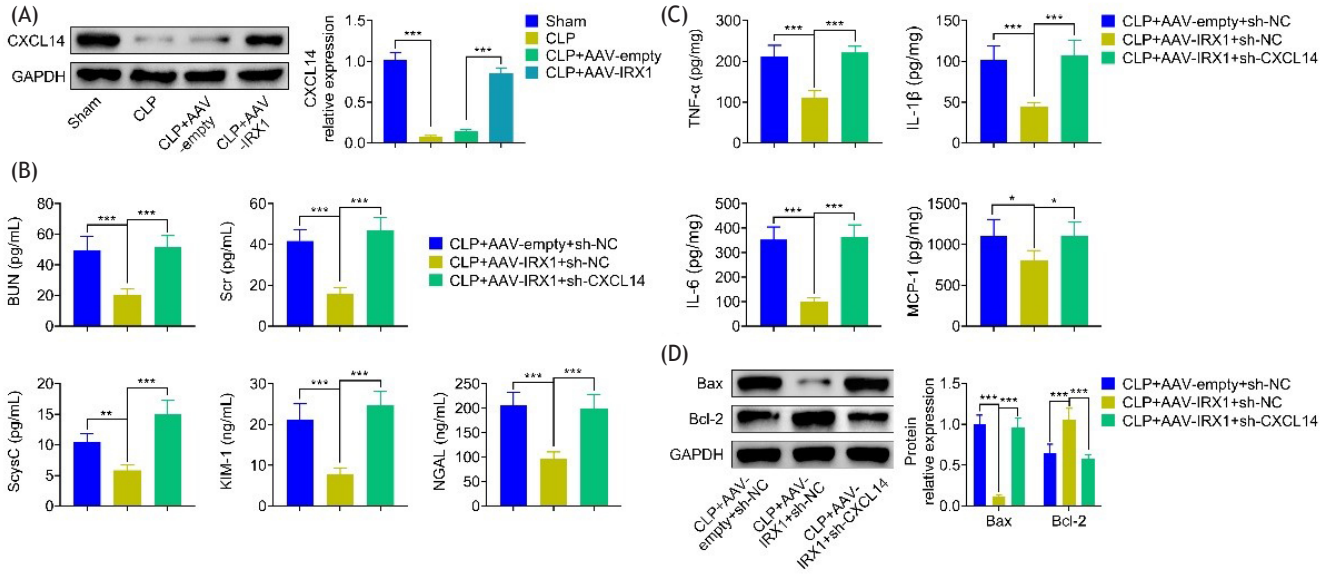

Figure 5 *IRX1*-modulated CLP-induced kidney injury in mice via CXCL14 activation. (A) The protein expression of CXCL14 was examined by Western blot analysis. (B) The expression levels of Scr, ScysC, and BUN were measured by an automatic biochemical analyzer. The expression levels of NGAL and KIM-1 were measured by commercial kits. (C) The expression levels of inflammatory cytokines in blood, including TNF-α, IL-6, MCP-1, and IL-1β, were measured by commercial ELISA kits. (D) The expressions of apoptosis-related proteins were measured by Western blot analysis. *P < 0.05, **P < 0.01, and ***P < 0.005 vs. sham or IRX1 + AAV-empty group. Data were expressed as mean ± SEM.

to combat human body's infection, excessive abnormal inflammatory response can lead to the nonspecific killing of self-organized cells. In sepsis pathology, inflammatory response is like a double-edged sword. During sepsis, inflammation serves as an essential step in the fight against pathogens, and this immune response is usually strictly controlled to avoid overactive immune cells.[27] However, intracellular signaling processes are complex, and pathogens often lead to excessive inflammation in the early stages of sepsis, including subsequent multi-organ dysfunction syndrome, and hypotension resulting in inadequate renal perfusion and renal dysfunction.[28] A recent study has revealed that suppressed release of pro-inflammatory cytokines, including IL-6 and TNF-α, and reduced bacterial load in septic mice, contributed to the improved survival of mice.[29] Thus, the focus of this study was to suppress excessive inflammation. Our data indicated that AAV-IRX1 treatment attenuated the expressions of inflammatory cytokines under CLP condition. A previous study has shown that apoptosis appeared in renal diseases.[30] Monocytes or macrophages secrete pro-inflammatory cytokines, including TNF-α and nitric oxide (NO), both are factors that increase apoptosis. The use of immune-mediated stimulants, such as TNF, triggers apoptosis in the sepsis of acute kidney injury. In the present work, these data supported a protective role of *IRX1* in mediating the regulation of CLP-induced cellular apoptosis, and improving the survival rate.

Another recent study on osteosarcoma revealed that downregulation of *IRX1* in osteosarcoma cell lines caused the inhibition of CXCL14, reduction of NF-κB activity, and suppression of metastasis.[31] Interestingly, CXCL14 showed bactericidal activity against Gram-positive cocci and virulent pathogens;[32] in addition, it also had antimicrobial effects against *Escherichia coli* and *Staphylococcus aureus*. Meanwhile, CXCL14 demonstrated chemoattractant effects on immune and inflammatory cells.[33] However, no mechanism related to the regulation of CXCL14 expression by *IRX1* has been reported previously. This research was the first to show that CXCL14 expression was remarkably suppressed in mice upon CLP procedure, and treatment of AAV-IRX1 reversed this effect. Moreover, CLP + AAV-IRX1 treatment ameliorated the expressions of inflammatory cytokines and weakened the rate of cellular apoptosis, which was also disrupted in the CLP + AAV-IRX1 + sh-CXCL14 group. These results highlighted that *IRX1* exerted a protective influence on CLP-induced acute kidney injury by suppressing CXCL14 activation.

Conclusion

In conclusion, these results suggested that overexpression of *IRX1* promoted survival rate and suppressed CLP-induced apoptosis, inflammatory response, and kidney dysfunction. Furthermore, the protective effect of *IRX1* was partly generated by the activation of CXCL14. *IRX1* and CXCL14 were essential for elucidating the mechanism of acute kidney injury, and these findings could help to identify promising targets for clinical sepsis therapy.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Competing Interests

The authors stated that there were no conflicts of interest to disclose.

Author Contributions

Jie Zhang designed the study, completed the experiment, and supervised data collection. Yanlin Yue analyzed and interpreted the data. Yunyan Ma prepared the manuscript for publication and reviewed its draft. All authors read and approved the final manuscript.

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